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(57) Abrégé/Abstract:
The invention relates to a method for analysis of methylation models in genomic DNA. In contrast to conventional methods for the analysis of methylation models, said method comprises a two-part method, whereby the genomic DNA is firstly subjected to a methylation specific treatment followed by an isothermal replication step.

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(54) Title: METHOD FOR ANALYSIS OF GENOMIC METHYLATION MODELS

(54) Bezeichnung: VERFAHREN ZUR ANALYSE GENOMISCHER METHYLIERUNGSMUSTER

(57) Abstract: The invention relates to a method for analysis of methylation models in genomic DNA. In contrast to conventional methods for the analysis of methylation models, said method comprises a two-part method, whereby the genomic DNA is firstly subjected to a methylation specific treatment followed by an isothermal replication step.

(57) Zusammenfassung: Die Erfindung offenbart ein Verfahren zur Analyse von Methylierungsmustern in genomischer DNA. Im Unterschied zu gegenwärtigen Verfahren der Analyse von Methylierungsmustern umfasst es eine zweiteilige Methode, bei der genomische DNA zuerst einer methylierungsspezifischen Behandlung ausgesetzt wird, der ein isothermer Replikationschritt folgt.

Method for analysis of genomic methylation models

Field of the invention

The invention relates to a method for the analysis of methylation patterns within genomic DNA. The invention discloses a method for the analysis of methylated cytosines. This is of particular interest as aberrant cytosine methylation patterns within genomic DNA have been associated with a variety of disease.

Prior Art

The most common covalent modification of genomic DNA is the methylation of cytosine to 5 methylcytosine. Cytosine methylation plays an important role in gene expression and regulation and has been shown to be critical in the maintenance of normal cellular functions. It is associated with genomic imprinting, embryonic development and a wide variety of diseases, including cancer.

For example, aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. Cancer Res 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., Curr Top Microbiol Immunol 249:75-86,2000). Using restriction landmark genomic scanning, Costello and coworkers were able to show that methylation patterns are tumour-type specific (Costello, J. F., et al., Nat Genet 24:132-138, 2000). Highly characteristic DNA methylation patterns could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet 8:459-470, 1999). Genome wide assessment of methylation

status represents a molecular fingerprint of cancer tissues.

The identification of 5-methylcytosine as a component of genetic information is therefore of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing development as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

Currently the most frequently used method for analyzing DNA for 5-methylcytosine is based upon the reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behaviour. However, 5-methylcytosine remains unmodified under these conditions.

Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization or sequencing. Particularly favoured is the amplification of treated sequence followed by analysis of the CG and TG dinucleotides within the amplificate.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 1997 Mar-Apr;5(2):94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either

completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. 1997 Jun 15;25(12):2529-31, WO Patent 9500669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 1997 Jun 15;25(12):2532-4). In addition, detection by hybridization has also been described (Olek et al., WO 99 28498).

Compared to sequencing, bisulfite analysis is a relatively more precise method of quantifying the degree of methylation at a particular target. However, the technique is limited by the difficulty of carrying out the simultaneous analysis of multiple CpG targets within a target nucleic acid, for example by multiplex PCR.

Methods for the amplification of specific DNA targets are based upon template directed primer extension by polymerases. The most widely utilised of these methods is the polymerase chain reaction 'PCR' (Mullis, K. et al., Cold Spring Harbor Symp. Quant. Biol. 51:263-273 (1986); Erlich H. et al., EP 50,424; EP 84,796, EP 258,017, EP 237,362; Mullis, K., EP 201,184; Mullis K. et al., U.S. Pat. No. 4,683,202; Erlich, H., U.S. Pat. No. 4,582,788; Saiki, R. et al., U.S. Pat. No. 4,683,194 and Higuchi, R."PCR Technology," Ehrlich, H. (ed.), Stockton Press, NY, 1989, pp 61-68).

In the polymerase chain reaction successive cycles of denaturation are followed by annealing and polymerisation. In the first step the DNA double helix is denatured by

transient heating. This is followed by the annealing of two species of primers, one to each strand of DNA. Subsequently the annealed primers are extended using a polymerase. This is followed by the denaturation of the resultant double stranded nucleic acids, allowing each strand to serve as a template for another cycle of template directed primer extension.

Although widely utilised and highly sensitive, one major drawback of the PCR is the difficulty of quantifiably simultaneously amplifying multiple fragments using multiple primers (multiplex PCR). A number of factors contribute to the problem, principally:

1. The priming efficiency of each species of primer is likely to be different, in part this is due to stability and structural differences between primers.

2. The denaturation rate of each primer target within the template nucleic acid may differ, therefore the rate of primer annealing will also differ between each site.

3. Due to the exponential nature of the PCR, any slight difference in yield between species of primers is amplified with each cycle.

An alternative to PCR is isothermal DNA replication, in particular rolling circle replication, based upon a naturally occurring bacterial method of DNA replication.

In such a system, a circularised nucleic acid known as an amplification target circle (ATC) is isothermally replicated using rolling circle replication primers and a polymerase. There are no denaturation and annealing stages, hence DNA replication is both continuos and isothermal.

The resultant DNA comprises a catenated linear DNA of identical sequence to the ATC.

Several variants of the method have been described. U.S. Pat. No. 5,871,921 (Landegren et al.) describes one method in which rolling circle amplification may be used for detection of genomic variants. In the assay, a detectable nucleic acid probe is hybridized to a single stranded nucleic acid target. The hybridization of the probe to the nucleic acid only occurs if the target sequence is present. The hybridized probe ends are then covalently connected to form a continuous loop of probe nucleic acid. This is followed by the removal of uncircularised probe molecules e.g. by exonuclease digest. The target molecule may then be detected by determination of the presence of the interlocking catenated probe.

An alternative method of using the rolling circle amplification process is disclosed in U.S. Pat. No. 5,648,245, Fire et al. The reference describes a four-step process for generating a concatamer library. In the procedure, the first step is to generate an amplification target circle by annealing ends of a padlock probe to a target nucleic acid sequence followed by ligation of the ends of the padlock probe to form a continuous loop. Once the amplification target circle is formed, the second step is to create a single stranded tandem-sequence DNA by rolling circle amplification of the amplification target circle. The third step requires converting the single stranded tandem-sequence DNA to double stranded tandem-sequence DNA. Finally, the double stranded tandem-sequence DNA is cloned or used for in vitro selection.

U.S. Pat. No. 5,866,377 uses rolling circle amplification as a method to detect variants in a nucleic acid sequence. In this method, a padlock probe hybridizes to a

single stranded nucleic acid such that the ends are adjacent to each other. A ligation step is then carried out such that, in the presence of a specific variant base at the locus near the end base of one of the probe ends a ligase is able to join the ends of the probe molecule together to form a circularized molecule. Detection of the presence of the catenated probe on the target nucleic acid indicates the presence of the specific variant.

U.S. Pat. No. 5,854,033 (Lizardi) describes a similar assay where the catenated probe is used to produce tandem-sequence DNA by rolling circle amplification. The tandem sequence is detected to determine the amount of target sequence present.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., Nucleic Acids Res. 1998, 26, 2255.

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. Bioessays. 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet. 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression

in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97 46705, WO 95 15373 and WO 45560.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorophore labelled probe oligonucleotides are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

In addition to the simple detection of fluorescent labels, two other detection methods are often used in nucleic acid analysis. interaction between two molecules wherein the excited state of one molecule (the donor) transfers energy to the other molecule (the acceptor). The donor molecule is a fluorophore while the acceptor molecule may or may not be. The energy transfer occurs without the emission of photons, and is based on dipole-dipole interactions between the two molecules. Molecules that are commonly used in FRET include fluorescein, N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL), and 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS).

A further detection method for the analysis of biomolecules using fluorescent molecules is the use of fluorescence polarisation. Fluorescence polarisation relies

on the property of plane polarised light to be emitted by a stationary fluorescent molecule. If plane polarised light is used to irradiate a fluorescent molecule, the molecule will emit plane polarised light between excitation and emission only when stationary. Larger molecules, i.e. those of larger molecular weight and/or volume tumble more slowly about their axes than smaller molecules. The application of FP techniques to nucleic acid analysis is disclosed in patent application EP 0382433 B1.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapor phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the

selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallization. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. Nucleic Acids Res. 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989.

Description

The invention discloses a method for the analysis of methylation of CpG dinucleotides within genomic DNA. It presents advantages over the prior art in that it allows a quantitative analysis of the degree of methylation at a specific CpG site, or multiple sites, within a DNA sample. This is particularly significant wherein the methylation status analysis requires amplification of multiple

DNA fragments within a sample, which may not be quantifiably amplified using multiplex PCR.

In general the method comprises the following steps. Firstly genomic DNA is obtained from cellular or other sources. The DNA of interest (the target DNA) is then isolated from the genomic DNA. In the next step the isolated DNA may then undergo a methylation specific treatment resulting in the formation of single stranded DNA, such as bisulfite treatment, or methylation sensitive restriction enzyme digest (which may be combined with the previous step), followed by amplification of one of the two strands. This is followed by a circularisation step to form an amplification target circle which is then transcribed in a replication reaction using rolling circle replication primers and a polymerase. The replicated nucleic acid takes the form of a linear nucleic acid comprising catenated tandem copies of the amplification target circle sequence.

In the first step of the method genomic DNA is obtained from cellular, tissue or other sources using standard methods, as found in references such as Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989. The extracted DNA may then be fragmented using means standard in the art, such as, but not limited to, restriction endonuclease digest.

The second step of the method is a methylation specific treatment of the genomic DNA fragment that results in the formation of single stranded nucleic acids. In one embodiment of the method the treatment may be carried out using methylation sensitive endonucleases. It will be obvious to one skilled in the art that a methylation sensitive endonuclease digest may be carried out in a manner capable of discriminating between methylated and unmethy-

lated CpG dinucleotides, as illustrated by Costello, J. F., et al., Nat Genet 24:132-138, 2000. The digest may be carried out such that CpG islands within the sequence are left intact, for example using a restriction enzyme specific for the sequence TTAA. Alternatively, the digest may be carried out using restriction enzymes that digest within CpG islands, thus allowing methylation status analysis. In a further preferred embodiment this step may be combined with the previous step, wherein the DNA of interest is isolated by a methylation sensitive restriction enzyme digest.

Methylation sensitive digest is then followed by the amplification of the strand of interest, for example using asymmetric PCR. The resultant single stranded fragments are then ligated into a circular conformation by means of a ligation oligonucleotide in the third step of the method.

In an alternative embodiment of the method, the second step may take the form of a chemical treatment. The genomic DNA fragments may be treated such that cytosine bases within the DNA which are not methylated at the 5-position are converted to a base which has dissimilar base pairing properties. Nonetheless, cytosines which are methylated at the 5-position remain unchanged by the treatment. In a preferred embodiment the treatment is carried out using a bisulfite reagent (e.g. hydrogen sulfite, disulfite). An addition takes place at the non-methylated cytosine bases. Furthermore, a denaturating reagent or solvent as well as a radical scavanger are required to be present. A subsequent alkaline hydrolysis results in the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted single stranded DNA may then be used for the detection of methylated cytosines.

As each CG dinucleotide may be converted into a TG dinucleotide or remain unconverted, dependant on its methylation status, bisulfite treated CpG rich DNA has several unique characteristics. Firstly, the sense and antisense strands of the DNA are converted such that they are no longer complementary. In addition, bisulfite treatment of CpG rich DNA results in the creation of two species of strands, one relatively thiamidine rich and the other relatively cytosine rich. Furthermore, as each CG dinucleotide within the sense strand is hybridised to a CG dinucleotide on the antisense strand of DNA, each CG position may be analysed on both strands of the DNA.

In an alternative embodiment of the method, the treatment step of the method may comprise of a methylation sensitive restriction enzyme digest followed by a bisulphite treatment step.

In the third step of the method, the treated single stranded DNA is ligated into a circular conformation, by means of an oligonucleotide. Firstly the DNA is contacted with a specifically designed oligonucleotide, known as a ligation oligonucleotide, leading to the formation of a circular hybrid species of DNA which is subsequently ligated to form a covalently closed circular DNA.

The ligation oligonucleotide (illustrated in Figure 1) is linear single stranded DNA molecule with a 3' hydroxyl group and a 5' phosphate group, allowing the ends to be ligated using a DNA ligase. For the purposes of this invention the ligation oligonucleotide should comprise a 3' hydroxyl group a left target probe sequence, a spacer sequence, a right target probe sequence and a 5' hydroxyl group. The spacer sequence should include a primer complement region for binding of rolling circle replication

primers. The sequence of the spacer region should be such that it is not significantly complementary to any other sequence within the target DNA.

Hybridisation of the target probe regions to the single stranded target DNA molecule is carried out, whereby the left target probe region base pairs with the 5' end of the DNA fragment, and the right target probe region base pairs with the 3' end of the DNA fragment resulting in the formation of a circular hybrid nucleic acid.

In the fourth step of the method, the circularised nucleic acid is then contacted with a ligase under conditions conducive to ligation, such that the hybridised sections of the ligation oligonucleotide and the target nucleic acid are ligated to form a continuous circular nucleic acid (hereinafter referred to as an amplification target circle, or ATC), this is illustrated in Figure 2.

Circularised DNA fragments may then be isolated from linear ligation products by any means standard in the art, for example, but not limited to, gel electrophoresis and exonuclease digest.

In the fifth step of the method the ATC is replicated using a rolling circle technique.

Firstly, a rolling circle replication primer is contacted with the ATC, under conditions conducive to the annealing of the primer to the ATC. The primer should consist of a sequence complementary to the primer complement region. The sequence of the primer should be such that it is not significantly complementary to any other sequence within the circularised DNA. It is preferred that the primers used in the rolling circle replication contain an additional sequence at the 5' end that is non complementary

to any sequence within the circularised DNA. This sequence facilitates the displacement of the replicated DNA strand from the ATC.

Subsequent to the annealing of the primer to the ATC (illustrated in Figure 3) the ATC sequence is replicated into a continuous linear nucleic acid comprising tandem repeats of the ATC sequence (hereinafter referred to as tandem sequence DNA) . This is achieved by the addition of a DNA polymerase and nucleotides. DNA polymerase suitable for use in the disclosed method are required to be capable of rolling circle replication of primed single stranded circular DNA. Such polymerases are hereinafter referred to as rolling circle polymerases. It is preferred that the rolling circle polymerases do not have a 5' to 3' exonuclease activity, and that the polymerases are capable of displacement of the synthesised strand. Examples of suitable polymerases include the Klenow fragment of DNA polymerase I, phage M2 DNA polymerase, T4 DNA polymerase and bacteriophage .o/.29 DNA polymerase.

The nucleotides which are incorporated into the tandem sequence DNA may be labelled, allowing detection of the labels in the following step. Suitable labels are described below.

In further preferred embodiment of the invention strand displacement factors are added during the replication step of the method. Such factors include DNA helicases, such as calf thymus helicase or proteins such as single stranded DNA binding proteins and adenovirus DNA- binding protein.

In the final step of the method the tandem sequence DNA is detected.

In one embodiment of the method, the nucleotides incorporated during the ATC replication may be labelled with a detectable label allowing detection of nucleotides incorporated within tandem sequence DNA. A wide variety of molecules are suitable for use with this technique, for example, but not limited to, mass, fluorophore and radioactive labels.

In a further embodiment of the invention, detection may be carried out using labelled probe oligonucleotides. The probe oligonucleotides detect the presence of specific sequences within the tandem sequence DNA by hybridisation to the tandem sequence DNA. Such oligonucleotides are hereinafter referred to as detection oligonucleotides. The detectable labels may include, for example, but not limited to, mass, fluorescent (including FRET and fluorescence polarisation) and radioactive labels.

In a further embodiment of the method the rolling circle replication primers or oligonucleotide probes may be immobilised upon a solid phase surface with all subsequent steps of the method being carried out upon the solid phase surface. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices are possible as well.

Furthermore multiple primers may be immobilised upon the the solid phase surface in the form of an array allowing high throughput analysis of multiple DNA samples.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a

carrier material for analysis of the methylation status of a genomic DNA, in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of rolling circle replication primer oligonucleotides, oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

In a further embodiment of the method CpG methylation analysis may also be carried out using a specifically designed probe molecule.

Firstly, genomic DNA is obtained from cellular, tissue or other sources using standard methods, as found in references such as Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989. The extracted DNA may then be fragmented using means standard in the art such as, but not limited to, restriction endonuclease digest.

In the first step of the method the DNA is treated such that cytosine bases within the DNA which are not methylated at the 5-position are converted to a base which has dissimilar base pairing properties. Nonetheless, cytosines which are methylated at the 5-position remain unchanged by the treatment. In a preferred embodiment the treatment is carried out using a bisulfite reagent (e.g. hydrogen sulfite, disulfite). An addition takes place at the non-methylated cytosine bases. Furthermore, a denatu-

rating reagent or solvent as well as a radical scavanger are required to be present. A subsequent alkaline hydrolysis results in the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted single stranded DNA may then used for the detection of methylated cytosines.

In the second step of the method the target DNA is analysed using a nucleic acid probe hereinafter referred to as an open circle probe (OCP). The OCP is hybridised to the target DNA, then ligated to form a covalent loop followed by rolling circle replication.

The probe is similar to the ligation oligonucleotide described earlier (illustrated in Figure 1), it is a linear single stranded DNA molecule with a 3' hydroxyl group and a 5' phosphate group, allowing the ends to be ligated using a DNA ligase . The use of OCPs have been described, for example in U.S. Patent 6,210,884. For the purposes of this invention the probe should comprise a 3' hydroxyl group a left target probe sequence, a spacer sequence, a right target probe sequence and a 5' hydroxyl group. The spacer sequence should include a primer complement region for binding of rolling circle replication primers. Furthermore, the spacer region may comprise detection tag portions and promoter portions. In a preferred embodiment the OCP does not contain any self complementary regions. It is further preferred within OCPs containing a promoter portion that the OCP does not contain any sequences resembling a transcription terminator. The OCP may be immobilised on a solid surface with all further steps of the method carried out upon the surface, thus allowing for high throughput analysis of DNA samples.

Target probe sequences.

There are two target probe sequences, one at each end of the OCP. The target probe region at the 3' end of the probe is hereinafter referred to as the left target probe, whereas the target probe region at the 5' end of the probe is hereinafter referred to as the right target probe. Each target probe is complementary to a target sequence within the treated genomic DNA. The target probe sequences are complementary to adjacent target regions of the treated genomic DNA. In a preferred embodiment each target probe may contain one or more CG dinucleotides.

Upon hybridisation of the target probes to the treated genomic DNA the 3' hydroxyl group of the left target probe is adjacent to 5' phosphate group of the right target probe, allowing the two ends to be joined together by a ligation reaction resulting in the circularisation of the linear OCP.

The hybridisation of the open circle probe to the target DNA may be such that a gap, of one or more bases, is present between the 5' and 3' ends of the open circle probe. In such a case, the gap may be filled prior to, or during the ligation step. The gap may be filled by one or more oligonucleotides, or nucleotides, or a combination of both. These nucleotides and/or oligonucleotides may carry detectable labels, the use of which will be described later.

The ligated OCP, hereinafter referred to as an amplification target circle (ATC) is then replicated using a rolling circle technique, as has been described previously, using rolling circle replication primers and polymerases.

Figures

Figure 1

Figure 1 illustrates the basic structure of a ligation oligonucleotide or open circle probe, wherein A is the left target probe region, B is the right target probe region and C is the spacer region which includes a primer complement sequence.

Figure 2

Figure 2 illustrates the hybridisation of a a ligation oligonucleotide to the target nucleic acid resulting in the formation of a circularised nucleic acid.

Figure 3

Figure 3 illustrates the annealing of a rolling circle replication primer to the amplification target circle. 'A' represents the left target probe region of the ligation oligonucleotide which is ligated to the 5' end of target DNA (E), 'B' represents the right target probe region of the ligation oligonucleotide which is ligated to the 5' end of target DNA (F). C is the rolling circle replication primer which is annealed to D, the spacer region of the ligation oligonucleotide.

Example:

Assay to detect the methylation status of the gene MDR 1

In the following example, rolling circle amplification was used to ascertain the methylation state of the CG dinucleotides within the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1) within the gene 'multidrug resistance' Genbank Accession Number NM_000927 (MDR1). In the first example (Example 1) an assay was established to detect the

presence of methylated versions of the gene in question, in the second example (Example 2) an assay was established to detect the presence of unmethylated versions of the gene. In the third example (Example 3) the two assays were combined in order to determine the degree of methylation within a sample.

DNA was extracted from tissue samples using a Qiagen extraction kit according to manufacturer's instructions. The DNA from each sample was treated using a bisulfite solution (hydrogen sulfite, disulfite) according to the agarose bead method (Olek et al 1996). The treatment is such that all non methylated cytosines within the sample are converted to thiamidine, conversely 5-methylated cytosines within the sample remain unmodified.

Example 1

Detection of methylation within the multidrug resistance gene

The following example describes the detection of the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1)within the multidrug resistence (MDR1) gene in its CG-methylated state.

Hybridization and circularization of ligation oligonucleotide (open circle probe)

Following isolation and bisulphite treatment of the genomic sequence according to the means described above, the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1) was converted to TAGGAATAGCGTCGGGGCGTGGGT (SEQ ID NO 2).
The following reaction mixture was then combined in a PCR tube:

10 µl of bisulfite treated target DNA within 50 mM Tris-HCl (pH 8.3) is added to 10 µl of a ligation reaction mixture containing:

1.5 U of Ampligase thermostable DNA ligase (Epicentre Technologies)

10 mM $MgCl_2$

50 mM KCl

0.02% Triton-X-100

1 mM nicotinamide adenine dinucleotide (NAD) is added.

After addition of 1µl of a 2 µM solution of the ligation oligonucleotide (open circle probe) 5´-phosphoryl-ACGCTATTCCTATTAGAGACTAGTGTTCTACTAATGTGAATCGATGAGTTAATATTT TACCCACGCCCCG-3´-OH (SEQ ID NO 3) the ligation mixture was incubated in an Eppendorff thermocycler at 95°C for 3min followed by 30 min at 65°C and finally at 4°C for up to 5 hours until further use.

Hybridization of primer and rolling circle amplification

To the ligation mixture 5 µl of a 0.6 µM solution of RCA primer 5´- CATTAGTAGAACACTAGT -3´ (SEQ ID NO 4) was added. After incubating at 70°C for 5 min and cooling to room temparature 5 µl of RCA reaction mixture containing 800 nM dNTP´s, 50 mM Tris-HCl, pH 8.3, 25 mM ammonium sulfate and 5 U *Bst* DNA Polymerase large fragment (New England Biolabs) was added and the RCA mixture was incubated at 65°C for 1 h and stored at 4°C for further use.

Detection of RCA product

The RCA product is spotted onto polylysine glass slides (Fisher Scientific). After drying and washing with water the slide is hybridised with the fluorescently labelled oligonucleotide probe Cy5- ACCCACGCCCCGACGCTATTCCTA (SEQ ID NO 5). After washing off residual probe the slide is scanned in a fluorescence scanner (from the manufacturer

Axxon). The presence of CG methylation in the target sequence is reflected by the observation of an intense Cy5 fluorescence at the corresponding position.

Example 2

Detection of the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1) within the multidrug resistence (MDR-1) gene in its CG-nonmethylated state.

Hybridization and circularization of ligation oligonucleotide (open circle probe)

Following isolation and bisulphite treatment of the genomic sequence according to the means described above, the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1)was converted to TAGGAATAGTGTTGGGGTGTGGGT (SEQ ID NO 6).

The following reaction mixture was then combined in a PCR tube:

10 µl of bisulfite treated target DNA within 50 mM Tris-HCl (pH 8.3) is added to 10 µl of a ligation reaction mixture containing:

1.5 U of Ampligase thermostable DNA ligase (Epicentre Technologies)

10 mM $MgCl_2$

50 mM KCl

0.02% Triton-X-100

1 mM nicotinamide adenine dinucleotide (NAD) is added.

After addition of 1 µl of a 2 µM solution of the ligation oligonucleotide (open circle probe) 5´-phosphoryl-ACACTATTCCTATTAGAGACTAGTGTTCTACTAATGTGAATCGATGAGTTAATATTT TACCCACACCCCA-3´-OH (SEQ ID NO 7)the ligation mixture is incubated in an Eppendorff thermocycler at 95°C for 3 min followed by 30 min at 65°C and finally at 4°C for up to 5 hours until further use.

Hybridization of primer and rolling circle amplification

To the ligation mixture 5 µl of a 0.6 µM solution of RCA primer 5´-ACTAGTGTTCTACTAATG-3´ (SEQ ID NO 8)was added. After incubating at 70°C for 5 min and cooling to room temparature 5 µl of RCA reaction mixture containing 800 nM dNTP´s, 50 mM Tris-HCl, pH 8.3, 25 mM ammonium sulfate and 5 U *Bst* DNA Polymerase large fragment (New England Biolabs) was added and the RCA mixture was incubated at 65°C for 1 h and stored at 4°C for further use.

Detection of RCA product

The RCA product is spotted onto polylysine glass slides (Fisher Scientific). After drying and washing with water the slide is hybridised with the fluorescently labelled oligonucleotide probe Cy3- ACCCACACCCCAACACTATTCCTA (SEQ ID NO 9). After washing off residual probe the slide is scanned in a fluorescence scanner (from the manufacturer Axxon). The presence of CG methylation in the target sequence is reflected by the observation of an intense Cy3 fluorescence at the corresponding position.

Example 3

This example describes the detection of the relative level of CG methylation within the sequence CAGGAACAGCGCCGGGGCGTGGGC (SEQ ID NO 1) of the multidrug resistence (MDR-1) gene.

Hybridization and circularization of ligation oligonucleotide (open circle probe)

Following isolation and bisulphite treatment of the genomic DNA according to the means described above the following reaction was carried out in a PCR tube:
10 µl of bisulfite treated target DNA within 50 mM Tris-HCl (pH 8.3) is added to 10 µl of a ligation reaction mixture containing:
1.5 U of Ampligase thermostable DNA ligase (Epicentre Technologies)
10 mM $MgCl_2$
50 mM KCl
0.02% Triton-X-100
1 mM nicotinamide adenine dinucleotide (NAD) is added.
After addition of 1µl of a 2 µM solution of the ligation oligonucleotide (open circle probe5´-phosphoryl-ACACTATTCCTATTAGAGACTAGTGTTCTACTAATGTGAATCGATGAGTTAATATTTTACCCACACCCCA -3´-OH (SEQ ID NO 7)and 5´-phosphoryl-ACGCTATTCCTATTAGAGACTAGTGTTCTACTAATGTGAATCGATGAGTTAATATTTTACCCACGCCCCG-3´-OH (SEQ ID NO 3) the ligation mixture is incubated in an Eppendorff thermocycler at 95°C for 3 min followed by 30 min at 65°C and finally at 4°C for up to 5 hours until further use.

Hybridization of primer and rolling circle amplification

To the ligation mixture 5 µl of a 0.6 uM solution of RCA primer 5´-ACTAGTGTTCTACTAATG-3´ (SEQ ID NO 8)was added. After incubating at 70°C for 5 min and cooling to room temparature 5 µl of RCA reaction mixture containing 800 nM dNTP´s, 50 mM Tris-HCl, pH 8.3, 25 mM ammonium sulfate and 5 U *Bst* DNA Polymerase large fragment (New England Biolabs) was added and the RCA mixture was incubated at 65°C for 1 h and stored at 4°C for further use.

Detection of RCA product

The RCA product is spotted onto polylysine glass slides (Fisher Scientific). After drying and washing with water the slide is hybridised with an equimolar mixture of the fluorescently labelled oligonucleotide probes Cy5-ACCCACGCCCCGACGCTATTCCTA (SEQ ID NO 5) and Cy3-ACCCACACCCCAACACTATTCCTA (SEQ ID NO 9). After washing off residual probe the slide is scanned in a fluorescence scanner (e.g. from Axxon). The level of CG methylation in the target sequence is reflected by the ratio of the Cy5/Cy3 fluorescence intensities at the corresponding position.

Sequence Listings

<110> Epigenomics AG

<120> Method for Analysis of Genomic Methylation Models

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<213> Homo Sapiens

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<212> DNA
<213> Artificial Sequence

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ccacacccca 70

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<212> DNA
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<400> 8

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<210> 9
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
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<400> 9

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Claims

1. A method for the analysis of cytosine methylation within a genomic sample comprising the following steps:

a) isolating a target DNA from a genomic DNA sample

b) treating the DNA in a manner capable of distinguishing methylated from unmethylated cytosine bases

c) contacting the target DNA sequence with a ligation oligonucleotide under conditions conducive to the formation of circularised target DNA-ligation oligonucleotide nucleic acid hybrids

d) contacting the circularised target DNA-ligation oligonucleotide nucleic acid with a ligase under conditions conducive to the formation of a continuous circular ligated nucleic acid (hereinafter referred to as an ATC)

e) contacting the ATC with a primer oligonucleotide under conditions conducive to hybridisation

f) adding polymerase to the template DNA under conditions that promote the replication of the template DNA, wherein replication of the template DNA results in the formation of a linear nucleic acid comprising tandem repeats of the template DNA sequence

g) detection of the tandem sequence DNA .

2. A method according to Claim 1, characterised in that the ligation oligonucleotide comprises a single stranded linear nucleic acid comprising from 5' to 3', a 5' phosphate group, a right target probe, a spacer region, a left target probe and a 3' hydroxyl group.

3. A method according to Claims 1 and 2, characterised in that the sequence of the target probe regions of the ligation oligonucleotide comprise at least one CG dinucleotide.

4. A method according to Claims 1 through 3, wherein the ligation oligonucleotide hybridises to the single stranded treated DNA only if the specific CpG dinucleotides under analysis were in a specific methylation status within the genomic DNA.

5. A method according to Claims 1 through 4, wherein Step A comprises restriction endonuclease digest by at least one restriction enzyme.

6. A method according to Claims 1 through 4, characterized in that step b) comprises a methylation sensitive restriction enzyme digest followed by the amplification of 1 strand of the target DNA.

7. A method according to Claims 1 and 4, characterised in that step b) comprises a treatment such that methylated cytosine bases within the sample are converted into a base which has dissimilar base pairing properties.

8. A method according to Claims 1 and 4, characterised in that step b) comprises a methylation sensitive restriction enzyme digest followed by a treatment such

that methylated cytosine bases within the sample are converted into a base which has dissimilar base pairing properties.

9. A method according to Claims 7 and 8, wherein the treatment is carried out by means of a bisulfite solution.

10. A method for ascertaining genetic and/or epigenetic parameters within a genomic DNA sequence by analyzing cytosine methylations characterized in that the following steps are carried out:

a) treating the DNA in a manner capable of distinguishing methylated from unmethylated cytosine bases such that the resultant target DNA is single stranded;

b) the pretreated single stranded DNA is contacted with at least 1 open circle probe under conditions conducive to hybridisation;

c) open circle probes hybridised to the target DNA are ligated to form amplification target circles(ATC);

d) a primer oligonucleotide is contacted with the amplification target circle under conditions that promote primer oligonucleotide ATC hybridisation;

e) polymerase is added to the primer oligonucleotide ATC mixture under conditions that promote the replication of the ATC, wherein replication of the ATC results in the formation of tandem sequence DNA

f) detection of the tandem sequence DNA.

11. A method according to Claim 10, characterized in that step a) comprises a methylation sensitive restriction enzyme digest followed by the amplification of 1 strand of the target DNA.

12. A method according to Claim 10, characterised in that step a) comprises a treatment such that methylated cytosine bases within the sample are converted into a base which has dissimilar base pairing properties.

13. A method according to Claim 10, characterised in that step a) comprises a methylation sensitive restriction enzyme digest followed by a treatment such that methylated cytosine bases within the sample are converted into a base which has dissimilar base pairing properties.

14. A method according to Claims 12 and 13, wherein the treatment is carried out by means of a bisulfite solution.

15. A method according to claims 10 through 14, characterised in that the open circle probe comprises one or more CG dinucleotides.

16. A method according to claims 10 through 15,wherein upon hybridisation of the open circle probe to the pre treated genomic DNA a gap of one or more bases exists between the 5' and 3' ends of the open circle probe, said gap filled in by means of one or more species of oligonucleotides or nucleotides or a combination thereof.

17. A method according to claim 16, characterised in that the gap filling oligonucleotides and /or nucleotides carry a detectable label.

18. A method according to claims 1 through 9, wherein the ligation oligonucleotide is immobilised upon a solid phase.

19. A method according to claim 18, wherein multiple ligation oligonucleotides are immobilised upon a solid phase.

20. A method according to claims 10 through 17, wherein the open circle probe is immobilised upon a solid phase.

21. A method according to claim 20, wherein multiple open circle probes are immobilised upon a solid phase.

22. A method according to claims 18 through 21, wherein one or more nucleic acids are immobilised upon a solid phase in the form of an array.

23. A method according to claim 22, characterized in that the immobilised nucleic acids are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.

24. A method according to claims 18 through 23, characterized in that the solid phase is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.

25. A method as recited in one of claims 1 and 10, wherein at least one species of labelled nucleotides is incorporated into the tandem sequence DNA.

26. A method as recited in one of claims 1 and 10, wherein the detection step comprises the hybridisation of a labelled detection oligonucleotide or peptide nucleic acid (PNA)-oligomer.

27. The method as recited in claim 26, characterized in that the labels are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.

28. The method as recited in Claim 27, characterized in that the labels are detected in a mass spectrometer.

29. The method as recited in one of Claims 26 and 27, characterized in that the produced fragments have a single positive or negative net charge.

30. The method as recited in one of claims 27 through 29, characterized in that the detection is carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

31. A method according to claim 26, characterized in that the detectable labels are radionuclides.

32. A method according to claim 26, characterized in that the labels are fluorophore molecules.

33. A method according to claim 32, characterized in that more than one species of fluorophore labels are used.

34. A method according to claim 33, wherein two types of labels are used, one type being a donor fluorophore and the other type being an acceptor fluorophore, and

wherein the fluorescence resonance energy transfer (FRET) between the two types of molecules is monitored.

35. A method according to one of claims 32 and 33, characterized in that the fluorescence polarisation of the label(s) is/are measured.

36. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent, rolling circle replication primers, polymerases, as well as ligation oligonucleotides according to claim 1 or open circle probes according to claim 10.

FIG.1

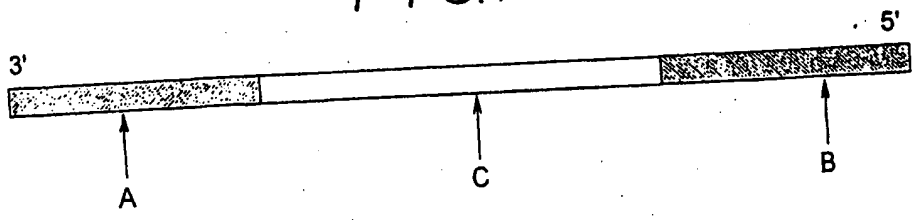

FIG. 2

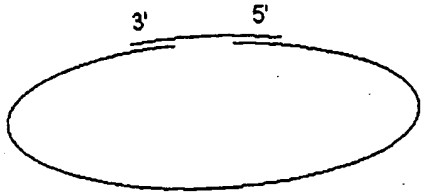

FIG. 3